Effects of effluent contamination of wetlands on population level changes in *Gambusia holbrooki*

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ABSTRACT

While presence/absence of endocrine disruption has been widely observed within polluted wetlands, relatively few data have addressed population level changes for any species. This paper investigated the effects of endocrine disruption on the phenotypic sex ratio, size structure, biomass, and density of *Gambusia holbrooki* populations in wetlands used for storage of 1) tertiary treated sewage effluent; 2) urban stormwater runoff; and 3) wetlands without effluent supplementation (control wetlands). Those wetlands that had previously been determined to have endocrine disruption effects on *G. holbrooki* had lower density and biomass of fish than other wetlands. In contrast, the pattern of variation in the average length and phenotypic sex ratio of fish was not consistent with the effects of endocrine disruption.

Key words: endocrine disruption, population structure, biomass loss, reduction in density, effluent pollution, mosquito fish

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Introduction

Water pollution from urban areas is typically derived from sewage effluent and urban runoff. Historically, pollutants from sewage effluent was considered to have the greatest detrimental effects in receiving bodies (Taebi and Droste, 2004), and urban runoff was the purview of engineers concerned with flood mitigation. However, in recent decades greater emphasis has been placed on the pollution load associated with urban runoff (Nix, 1994). With observations that have indicated that during peak flow times, urban runoff could have greater effects on the receiving waters than sewage effluent (Lee and Bang, 2000; Fletcher *et al*., 2008), there has been greater emphasis on its impact (e.g., Taebi and Droste, 2004; Chiew and McMahon, 1999; Drapper *et al*., 2000). A wide range of pollutants occur in sewage effluent (Alley, 2000) and stormwater (Burton and Pitt, 2001), encompassing physical, chemical, and biological contaminants. Such contaminants have been demonstrated to result in 'consistent declines' in richness of fish, invertebrate, and algal communities (Paul and Meyer, 2001). Some of these pollutants (e.g., sewage effluent – pharmaceuticals, plant sterols, sunscreens, Roberts and Thomas, 2006; Hogan *et al*., 2005: urban runoff – pesticides, herbicides, surfactants, heavy metals – Hogan *et al*., 2005) may interact with the endocrine systems of aquatic animals. Some of these pollutants (e.g., synthetic estrogens) that may have long-term sub-lethal effects on resident organisms (Roberts and Thomas, 2006) have been demonstrated to be Endocrine Disrupting Chemicals (EDCs, Hogan *et al*., 2005; cf. Endocrine Disrupting Compounds, Kavlock *et al*., 1996). Davenport and Davenport (2006) suggested that shallow confined areas such as estuaries and harbours, presumably compared to off-shore waters, are at greatest risk of problem level contamination. While boating and recreation may be influential in the transfer of chemical pollution (including EDCs) in coastal semi-enclosed waters, we consider that shallow, but smaller, impoundments used to store tertiary treated sewage effluent and/or stormwater runoff, would have a greater capacity to concentrate and store such pollutants.

Increasingly, there have been reports of developmental and reproductive impairment in wildlife. This has resulted in an intensification of studies into endocrine disruption (Hogan *et al*., 2005). Endocrine disrupting compounds have been defined as '*…exogenous agents that interfere with…natural hormones in the body responsible for the regulation of developmental processes*' (Kavlock *et al*. 1996, p. 716). The presence of such compounds in aquatic systems may affect a species' population attributes and result in a reduction in population size, feminisation, or occasionally masculinisation (Sumpter and Jobling 1995; Jobling *et al*. 1998; Taylor and

Harrison 1999). There is increasing awareness of the role that such aquatic pollutants play in the pathways of the endocrine system of aquatic vertebrates (Colborn and Thayer 2000; Hewitt and Servos 2001; Pickering and Sumpter 2003).

A recognised source of exposure to estrogenic endocrine disrupting compounds in aquatic species is through contact with contaminated waters. Such compounds may be of natural (e.g. estrogens; phytoestrogens) or synthetic (e.g. pharmaceuticals, phthalate plasticisers, surfactants, polychlorinated bi-phenyls [PCBs], polycyclic aromatic hydrocarbons [PAHs]; Rodriguez-Mozaz *et al.* 2004) origin, and they have been detected in waters receiving treated sewage (Jobling *et al.* 1998; Rodgers-Gray *et al.* 2001) and stormwater (Pickering and Sumpter 2003; Snyder *et al.* 2003) effluent.

Although the effects of endocrine disruption have been established in a range of aquatic species (e.g. alligators – Guillette *et al.* 1994; Crain *et al.* 1998; amphibians – Hemmingway *et al.* 2009; fish – van der Kraak *et al.* 1998; insects - Matthiessen *et al.* 1999; molluscs – Matthiessen and Gibbs 1998), there are few examples of population level changes including decline or extinction. In fish, sexual plasticity may occur in response to natural environmental effects and their ability to respond to such environmental challenges (e.g. increased egg production) may pre-adapt species to the challenges of endocrine disruption (Matthiessen 2000).

The Eastern Gambusia or Mosquitofish *Gambusia holbrooki* Girard 1859 has been a commonly used surrogate for the presence of endocrine disrupting compounds in freshwater systems (Jobling *et al.* 1998; Batty and Lim 1999; Toft *et al.* 2003; Norris and Burgin 2011a, b). The impacts of these compounds on population structure are less well understood. However there is evidence that such effects may occur. For example, Batty and Lim (1999) found that *G. holbrooki* density was lower at sites downstream from a treated sewage effluent outflow compared to an unaffected, upstream site. In wetlands used for holding ponds for either stormwater or treated sewage effluent, Norris and Burgin (2011a, b) found that characters of the gonopodium of *G. holbrooki* showed a morphological response that was consistent with endocrine disruption. These morphological challenges, particularly, the reduction in the length of the gonopodium, were sustained over time (Norris and Burgin unpubl. data). In this paper, the impacts on *G. holbrooki* populations resident in the wetlands used to store stormwater and treated sewage effluent were compared with farm dams (control wetlands) within the same area. We compared the phenotypic sex ratio, together with the size structure, biomass, and density of *G. holbrooki* populations across representatives of these wetland types. Our null hypothesis was that there was no statistically significant difference in phenotypic sex ratio, size structure, density, or biomass of *Gambusia holbrooki* resident in wetlands used to store treated sewage effluent, stormwater effluent, or wetlands without either contaminant.

Materials and Methods

Site Description

The study was carried out on the Hawkesbury campus of the University of Western Sydney, New South Wales, Australia (150°75' E, 33°62' S). This study site is located approximately 50 km northwest of Sydney's Central Business District (CBD) near Richmond in peri-urban northwestern Sydney. The area has a temperate climate and, during the year of the study, 2010, the mean monthly maximum temperature varied between 17.0°C (July) and 31.6°C (January). The annual precipitation was 713 mm. Mean monthly precipitation during the period varied between 16.2 mm (April) and 212.8 mm (February) (Bureau of Meteorology 2010).

The site had been used for agricultural teaching purposes for 120 years. Over this period dams were constructed to water grazing stock (Burgin 2010). Since the 1960s, tertiary treated sewage effluent from Richmond (population 2006 census = 5,560; ABS 2010) has been collected in purpose-built wetlands to 'polish' the water. This water was used principally for on-campus farm irrigation needs. More recently, the scheme was upgraded and expanded, and now includes wetlands constructed specifically to store stormwater from Richmond. These waters are also used for irrigation and, in addition, they are released to support environmental flows in the Hawkesbury – Nepean River. Both the treated sewage and stormwater effluent wetlands receive water from their respective sources in a specific sequence of movement through separate and discrete systems of wetlands (Fig. 1). These systems have a design working volume of 188.5 ML of tertiary treated sewage effluent, and 267.3 ML of urban stormwater runoff. The timing of the movement of water through these systems is based on the requirement to maintain water levels and is not based on specific retention time. The farm dams used as controls did not receive supplementary water from either effluent sources but relied on precipitation, predominantly rainwater, and ground water seepage (Norris and Burgin 2011a, b).

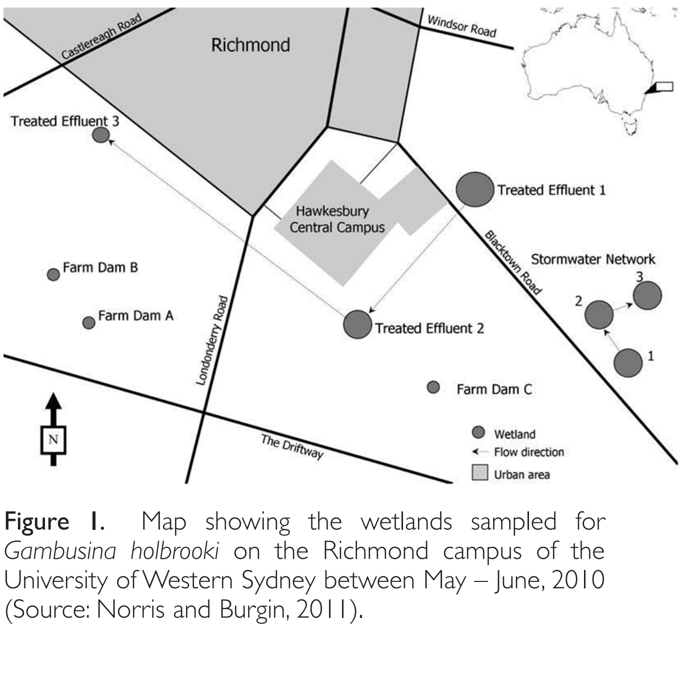

Figure 1. Map showing the wetlands sampled for *Gambusina holbrooki* on the Richmond campus of the University of Western Sydney between May – June, 2010 (Source: Norris and Burgin, 2011).

Sampling

Sampling was undertaken in May and July, 2010 on three separate occasions (i.e. three replications). Six of nine discrete wetlands sampled contained sufficient numbers of *G. holbrooki* to effectively sample: two received no water supplementation and relied on precipitation and ground water seepage (controls), two were supplemented with treated sewage effluent, and two relied on stormwater effluent. Sampling was therefore in two wetlands that received sewage effluent, two that received stormwater effluent supplementation, and two farm dams which received no water supplementation.

Sampling for phenotypic sex ratio and size structure

A 20 x 45 cm pool scoop on a 2 m telepole (3.8 m fully extended) was used to sample *G. holbrooki*. For the analysis of the phenotypic sex ratio and size distribution of fish among water sources, at each wetland, sampling was undertaken by progressively moving along the perimeter of the shore line until at least 60 adult fish had been collected or 2 hours of sampling had elapsed. Thirty juvenile *G. holbrooki* were also randomly selected from each of the wetlands. This random selection was achieved by retaining juveniles from every fifth dip net scope, or whenever they were collected if numbers were insufficient to obtain the sample size targeted. Non-target fish that were caught incidentally were immediately released at point of capture. Fish were placed in a container with approximately 5 L of water from the wetland from which they were netted and placed in shade until sampling at the wetland was completed. Once sufficient numbers were obtained, the fish were transported to the laboratory, euthanised and subsequently processed (c.f. Batty and Lim 1999; Norris and Burgin 2011).

Gambusia holbrooki were phenotypically sexed with the aid of an externally-illuminated dissecting microscope (Nikon, Lidcombe) at 40X magnification and a 50 mm glass slide ruler with divisions of 0.1 mm. The sex of adult fish was determined through visual morphological characters. For males, this was based on the elongation of the anal fin and the presence of developed terminal hooks. Females were considered mature when they possessed a gravid spot or were greater than 25 mm from the tip of the snout to the posterior of the mid-lateral portion of the hypural plate and did not possess the elongated anal fin rays of the adult male (cf. Norris and Burgin 2011). Juvenile *G. holbrooki* were also measured.

Sampling for biomass and density

To compare density and biomass among wetlands, sampling was undertaken three times between June and July 2010 (3 replicates). Sampling was restricted to areas associated with emergent vegetation. Preliminary sampling immediately before collection for the study revealed that few *G. holbrooki* were active in open waters. To maximise consistency of effort and habitat sampled, sampling was restricted to areas associated with emergent vegetation at the edge of wetlands.

In sampling, the same equipment and processing procedures were used as for comparing the size structure of the wetland populations except that, to ensure equivalent effort was expended in each wetland, all fish collected in 50 dip nettings were used as the basis of calculation.

In the laboratory fish were euthanised, blotted with a soft tissue to remove excess water, and weighed (Satrue, Taichung City; 0.01 accuracy) in batches of 20. This sub-sampling approach was used because 1) the accuracy of weighing wet fish (particularly fry) had the potential to introduce substantial bias; and 2) weighing hundreds of fish individually would be time consuming, and dehydration could exacerbate error. During the weighing process, the number of fish collected from each wetland was recorded so that the comparative density of *G. holbrooki* among wetlands could be analysed.

Water quality analysis

Water analysis was carried out with a portable 'Intelligent Water Quality Analyser' (Model 611, Yeo Kal Electronics Pty Ltd, Brookvale, Australia). In each wetland, replicate samples were taken monthly at three sites to assess commonly sampled physiochemical parameters (salinity, pH, temperature, dissolved oxygen, oxidation-reduction potential, turbidity).

Data analyses

To determine if phenotypic sex ratio differed among wetlands a Chi-square Goodness of Fit Analysis was undertaken. As a post hoc test, a G-test was used to investigate the phenotypic sex ratio among replicates within a wetland.

To analyse the size structure, and comparative density and biomass of *G. holbrooki* among water sources and wetlands, a Levene's Test was carried out to confirm normality before a 2-Way Nested Analysis of Variance (ANOVA) was conducted on the data. To establish where significant differences in fish biomass and density occurred among wetlands, a one-way ANOVA Post Hoc Sidak Test was used.

Regression analysis compared mean physiochemical water quality data for the months May, June and July 2010 (the month before fish sampling commenced and the period of sampling) to density and biomass of fish captured to investigate relationships between these parameters and fish population attributes.

Results

Phenotypic sex ratio

Using a Chi-square Goodness of Fit statistic, there was, overall, a significant difference from the expectation of a 1:1 phenotypic sex ratio ($\chi^2_5 = 255.06$, $p = < 0.0001$) in *G. holbrooki*. In five of the six wetlands sampled there was a bias towards males. One wetland (control 2) had an overall bias towards females. When the differences within and between sampling periods (replicates) were investigated using a G-test, in all replicates in the five wetlands with a significant difference from a 1:1 sex ratio, male abundance was greater than female. However, the wetland with the female fish bias showed inconsistent

Table 1. G-test results of sex ratio of replicate samples of *Gambusia holbrooki* from wetlands on the Hawkesbury campus, University of Western Sydney. Water was derived from treated sewage effluent [sewage], stormwater [stormwater], and farm dams without water supplementation [control]. Sampling was undertaken over May and June, 2010 (Significance levels * = significance = 0.05; ** = 0.01; *** = 0.001; ns = not significant

Replicate	Male	Female	Total	d.f.	G	P
Replicate 1						
Control 1	45	15	60	1	15.70	< 0.001***
Control 2	22	38	60	1	4.32	< 0.025*
Sewage A	19	3	22	1	12.97	< 0.001***
Sewage B	38	1	39	1	44.76	< 0.001***
Stormwater A	40	9	49	1	21.19	< 0.001***
Stormwater B	37	23	60	1	3.30	< 0.05*
Total for replicate				6	102.24	< 0.001***
Sum/Pooled	201	89	290	1	44.40	< 0.001***
Heterogeneity				5	57.84	< 0.001***
Replicate 2						
Control 1	52	8	60	1	36.06	< 0.001***
Control 2	21	22	43	1	0.02	> 0.05 ns
Sewage A	22	1	23	1	23.66	< 0.001***
Sewage B	55	5	60	1	48.76	< 0.001***
Stormwater A	26	12	38	1	5.28	< 0.01**
Stormwater B	45	15	60	1	15.70	< 0.001**
Total for replicate				6	129.47	< 0.001***
Sum/Pooled	221	63	284	1	93.11	< 0.001***
Heterogeneity				5	36.36	< 0.001***
Replicate 3						
Control 1	49	11	60	1	26.01	<0.001***
Control 2	14	5	19	1	4.44	<0.025*
Sewage A	28	4	32	1	20.25	<0.001***
Sewage B	59	1	60	1	73.01	<0.001***
Stormwater A	14	5	19	1	4.44	<0.025*
Stormwater B	49	11	60	1	26.01	<0.001***
Total for replicate				6	154.15	<0.001***
Sum/Pooled	213	37	250	1	136.96	<0.001***
Heterogeneity				5	17.19	<0.001***

results between samples (Table 1). On the first sampling occasion (replicate 1), there was a significantly higher abundance of females than males; on a separate occasion (replicate 2) there was no significant difference in sex ratio, and on the final sampling occasion (replicate 3) there were significantly more males than females.

Length

Overall the length of fish ranged from 10.5 to 35.0 mm (n = 1363). Although the two treated sewage effluent wetlands had the smallest average size fish, there was no significant difference among wetlands (Fig. 2).

Biomass

There was no significant difference in the biomass of fish among water sources ($F_{2, 17}$ = 1.766, p = 0.311); however, there was among wetlands ($F_{5, 17}$ = 5.500, p = 0.013, < 0.05; Table 2). The two wetlands that stored treated sewage effluent and the first in the chain of stormwater effluent wetlands were not significantly different from each other. Control wetlands and the second in the chain of stormwater effluent were also not significantly different from each other; however, there was a significant difference between the two sets of wetlands (Fig. 3).

Density

There was no significant difference in the density of fish among water sources ($F_{2, 17}$ = 1.091, p = 0.441); however, there was among wetlands ($F_{5, 17}$ = 7.749, p = 0.004, < 0.01; Table 3). One of the farm dams (control 1) and the second in the chain of stromwater effluent wetlands were not significantly different from each other. However, only

Table 2. Results of the analysis of a fully nested Analysis of Variance of biomass (gm) of *Gambusia holbrooki* to investigate pollution (treated sewage effluent, stormwater effluent, farm dams as controls) collected from wetlands on the Hawkesbury campus, University of Western Sydney. Sampling occurred between May - July 2010

Source	Degrees of freedom	Sum on squares	Mean sum of square	F-value	Probability
Factor A - Water Source	2	5944.613	2972.314	1.766	0.311
Factor B - Wetland	3	5049.503	1683.168	5.500	0.013
Error	12	3672.689	306.057		
Total	17	14666.818			

Table 3. Results of the analysis of a fully nested Analysis of Variance of density (gm) of *Gambusia holbrooki* to investigate pollution (treated sewage effluent, stormwater effluent, farm dams as controls) collected from wetlands on the Hawkesbury campus, University of Western Sydney. Sampling occurred between May - July 2010

Source	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	Probability
Factor A - Water Source	2	188809.000	94404.500	1.091	0.441
Factor B - Wetland	3	259693.000	86564.333	7.749	0.004
Error	12	134052.000	11171.00		
Total	17	582554.000			

this farm dam was significantly different from all other wetlands (Fig. 4).

Variation in water quality

A summary of factors that typically influence fish populations (salinity, dissolved oxygen, pH, water temperature, oxidation reduction potential, turbidity) are presented in Table 4. A quadratic equation provided the best representation of the association between the water quality parameters and both biomass and density. However, all parameters showed, at best, a very weak and non-significant relationship (Table 5).

Discussion

Variation in biomass and density among wetlands

Diniz *et al.* (2005) predicted that endocrine disruption would have an influence on density and biomass, and mortality. We found that biomass of *G. holbrooki* was lowest in the first in the series of wetlands that received either treated sewage or stormwater effluent. Compared to all other wetlands sampled, density was highest in one of the farm dams. The second in the sequence of stormwater wetlands was intermediate between this farm dam and other wetlands. This outcome was broadly similar to the pattern of gonopodium deformity that Norris and Burgin (2011a, b) observed in the same wetlands. Undertaking a qualitative study using gonopodium characters as a surrogate for endocrine disruption, they observed the greatest effect in the first in the sequence of wetlands to receive effluent (sewage or stormwater). Deformity of gonopodium in *G. holbrooki* in the second stormwater wetland was less than in the first of either effluent type, and deformity was mildest in the second treated effluent wetland. One of the farm dams also showed the same milder level of gondopodium deformity as observed in the second sewage effluent wetland. The biomass and density of *G. holbrooki*, therefore, shows a similar

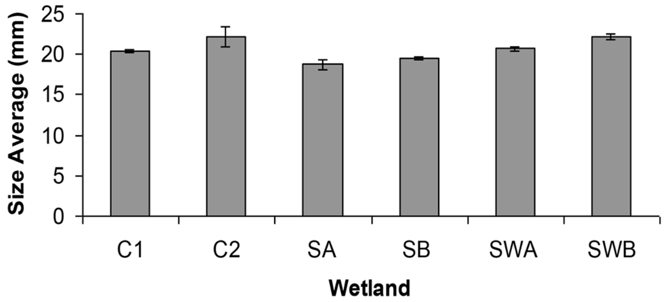

Figure 2. The average length (mm) of *Gambusia holbrooki* caught between May - June, 2010 from wetlands that received effluent (treated sewage, stormwater) and farm dams (controls, without water supplementation) on the Hawkesbury campus of the University of Western Sydney. C1 = Control 1, C2 = Control 2; SA = Sewage effluent A, SB = Sewage effluent B; SWA = Stormwater effluent A, SWB = Stormwater effluent B

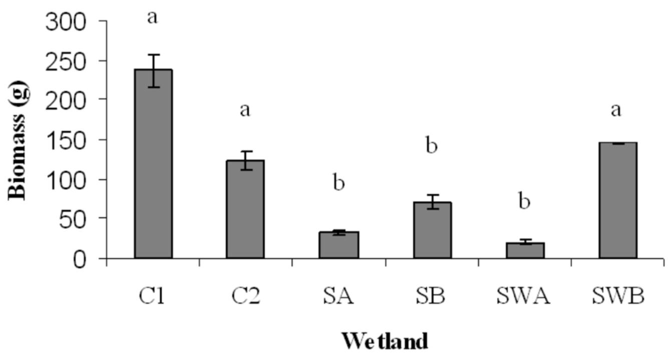

Figure 3. Biomass (g) of *Gambusia holbrooki* captured in June - July, 2010 from wetlands that received effluent (treated sewage, stormwater) and farm dams (controls, without water supplementation) on the Hawkesbury campus of the University of Western Sydney. C1 = Control 1, C2 = Control 2; SA = Sewage effluent A, SB = Sewage effluent B; SWA = Stormwater effluent A, SWB = Stormwater effluent B. Wetlands denoted with the same letter highlights that there is no statistically significant difference among these wetlands

Table 4. Summary of water quality data (water temperature, dissolved oxygen, pH, salinity, turbidity, oxidation reduction potential [oxidation reduction]) collected from wetlands on the Hawkesbury campus, University of Western Sydney. Sampling occurred between May - July 2010, to coincide with *Gambusia holbrooki* sampling of wetlands with stored treated sewage effluent [sewage], stormwater effluent [s'water], and farm dams without water supplementation [Control]

	Mean(se)	Range	Mean(se)	Range	Mean(se)	Range
Wetland	Temperature (°C)		Dissolved Oxygen (mg/L)		pH	
Control 1	11.2(1.0)	9.6-13.1	8.6(0.1)	7.2-10.4	7.6(0.1)	7.5-7.6
Control 2	9.2(1.2)	7.9-11.7	2.42(1.1)	0.3-3.8	7.1(0.6)	6.4-8.2
Sewage A	13.6(1.3)	11.1-16.1	10.20(1.4)	8.3-12.9	8.9(0.5)	7.8-9.5
Sewage B	13.6(1.2)	12.1-16.0	9.27(0.5)	8.3-10.0	9.0(0.0)	8.9-9.1
S'water A	12.4(1.0)	11.0-14.4	8.43(0.7)	7.3-9.8	7.9(0.0)	7.9-8.0
S'water B	12.1(0.9)	10.9-13.7	8.13(0.4)	7.6-8.8	8.1(0.3)	7.8-8.6
	Salinity (ppt)		Turbidity (ntu)		Oxidation Reduction (mV)	
Control 1	0.1(0.0)	0.1-0.1	116.3(40.0	72.9-196.2	79.8(6.5)	73.0-92-92.6
Control 2	0.1(0.0)	0.1-0.2	66.9(15.6)	41.9-95.4	20.7(14.1)	1.6-46.76
Sewage A	0.4(0.0)	0.4-0.4	21.3(7.1)	10.9-34.8	150.1(15.5	121.2-174.0
Sewage B	0.5(0.0)	0.5-0.5	54.6(10.9)	33.5-69.7	90.4(21.7)	49.4-123.0
S'water A	0.3(0.0)	0.3-0.3	66.3(25.5)	31.6-116.0	53.6(26.4)	4.6-95.2
S'water B	0.3(0.0)	0.3-0.4	105.0(47.5	11.5-166.4	154.3(10.8	144.0-176.0

Table 5. Comparison physiochemical water quality parameters and *Gambusia holbrooki* collected from wetlands with and without evidence of endocrine disruption on the Hawkesbury campus, University of Western Sydney. Sampling was undertaken between May-July 2010. No comparisons were statistically significantly different.

Water quality parameter	Density		Biomass	
	R	p-value	R	p-value
Salinity	0.2746	0.0901	0.2710	0.0934
pH	0.0945	0.4751	0.0544	0.6571
Temperature	0.0675	0.5920	0.0635	0.6114
Dissolved Oxygen	0.1086	0.4223	0.0782	0.5429
Oxidation Reduction Potential	0.2144	0.1637	0.2000	0.1877
Turbidity	0.1548	0.2834	0.2459	0.1204

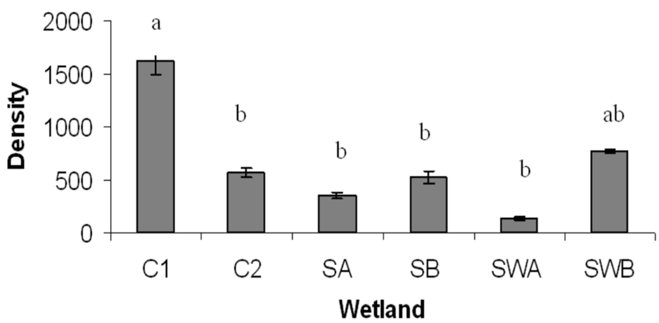

Figure 4. Density of *Gambusia holbrooki* captured in June - July, 2010 from wetlands that received effluent (treated sewage, stormwater) and farm dams (controls, without water supplementation) on the Hawkesbury campus of the University of Western Sydney. C1 = Control 1, C2 = Control 2; SA = Sewage effluent A, SB = Sewage effluent B; SWA = Stormwater effluent A, SWB = Stormwater effluent B. Wetlands denoted with the same letter highlights that there is no statistically significant difference among these wetlands

pattern of distribution of deformity of gondopodia as Norris and Burgin (2011a, b) had found. In wetlands where they reported the most extreme deformity of the gonopodium, biomass and density were lowest whereas were gonopodium deformity was mildest, biomass and density were highest.

Aquatic biological parameters may be strongly influenced by urban pollution (Lenat and Crawford 1994; Davies *et al.* 2010). The results of this study were not consistent with a detrimental impact due to such factors. However, *G. holbrooki* is acknowledged to have the ability to survive in a wide range of water conditions encompassing waters polluted by stormwater and treated sewage effluent (Norris and Burgin 2011a, b). Their widespread use as a test for environmental exposure to such contaminants is an example of their resilience to water pollution (Batty and Lim 1999; Toft *et al.* 2003; Game *et al.* 2006; Leusch *et al.* 2006).

4.2 Size structure of populations

Normal growth may be retarded in the presence of endocrine disruption (Game *et al.* 2006). Batty and Lim

(1999) observed that *G. holbrooki* were smaller in a river reach that received treated sewage effluent compared to upstream. Such variation has also been observed elsewhere. For example, Norris and Burgin (2011a, b) collected adult *G. holbrooki* in the study wetlands, and found differences in the average length of adult fish among wetlands that was consistent with endocrine disruption, and provided evidence that treated sewage effluent had an effect. Game *et al.* (2006) also observed that fish exposed to endocrine disruptors were smaller than those without exposure to contamination. Although distinct differences in average size among wetlands were not observed in this study, those that had been exposed to treated sewage effluent were of smaller average size than in other wetlands. The pattern is, therefore, consistent with the observations of others.

Growth, development and maturation vary with food availability and temperature, and thus season (Vondracek *et al.* 1988). Differences in these parameters may, therefore, occur over time. Toft *et al.* (2003) observed such length variation in *G. holbrooki*. In summer and early autumn (breeding season), Batty and Lim (1999) sampled within the same river catchment and found variation in length over the study. Norris and Burgin (2011a, b) sampled during summer, whereas the current study was undertaken in late autumn and winter (non-breeding season). The lack of clear variation in average length could have been due to the timing of fieldwork.

Time to maturity varies substantially within the year and could influence the size structure of *G. holbrooki* populations in different seasons. At low temperature, maturation of male fry may take 8 months, although more typically the juvenile phase varies between 18 and 56 days. Females may also take 8 months to mature although, more generally, this period is 18-70 days (Pyke 2005). There may also be differential mortality rates (Snelson 1989) which could affect the size structure of populations. Seasonal difference was, therefore, a likely explanation for the variation within and between sites over time, and differences observed among studies. Differences may also be due to microhabitat segregation. For example, distinct population 'systems' may occur in the field dependent on whether *Gambusia* spp. are sampled from open water or within vegetation (Martin 1975). However, since the current survey was carried out in cooler seasons there was effectively no fish activity in the open waters and so sampling was undertaken in equivalent habitat across this study but was not necessarily compatible with previous studies in terms of seasons and/or climatic conditions sampled.

Male bias in phenotypic sex ratio

Due to seasonal differences in growth and maturation (Trendall 1982), the phenotypic sex ratio of mature *G. holbrooki* could differ seasonally. Although in this study there was generally a bias towards males across wetlands, in one wetland the results varied among sampling episodes, and there was an overall bias towards females. This could have been due to sampling bias, although the excess of males in all other sampling periods and wetlands weakens such an explanation.

Mature males and females tend to grow at the same rate (1-2 mm/week) and generally mature at approximately the same age. Individuals of both sexes of a particular cohort would, therefore, be expected to mature simultaneously. However, the rate of development and, therefore, maturation time may differ between the sexes if microhabitat differences resulted in altered physical, chemical, and/or biological factors (e.g. water temperature, salinity, diet, density of conspecifics; Pyke 2005). Since the same habitat was sampled in each wetland, and *G. holbrooki* were effectively absent from open waters, it is assumed that resident fish were aggregated in edge habitat. The observation that water temperature, salinity and oxidation reduction potential (an indirect measure of food availability) were, at best, very weakly correlated with any population attribute, there was no evidence that these parameters were responsible for an apparent deficit of females in most wetlands.

However, it was observed that in the wetland where females were in highest proportion, physiochemical attributes (including temperature), tended to be the lowest sampled at any wetland (see Table 4). In the one wetland where females were a higher proportion of the catch, the density of fish was intermediate and, despite substantial differences in density in other wetlands, there were consistently a larger number of males than females across these wetlands on each sampling occasion. It is, therefore, unlikely that the bias observed was due to the density of conspecifics.

Norris and Burgin (2011a, b) determined that feminisation (specific deformities of the gonopodium consistent with endocrine disruption) was present in some wetlands sampled in the current study. Although endocrine disruptors may also masculinise fish (Bortone and Cody 1997), there was no evidence that this was occurring. *Gambusia affinis* females affected by endocrine disruptors have been found to have both the developed gonopodium consistent with the male of the species, and a gravid spot (Howell *et al.* 1980). No such individuals were observed in the present study, and these abnormalities have only been identified in effluent high in phytosterols, typically associated with paper mill waste effluent (Drysdale and Bortone 1989; Bortone and Cody 1997; Parks *et al.* 2001; Toft *et al.* 2004), and not stormwater or treated sewage effluent, the pollutants of our study.

Orlando *et al.* (2004) found that male fathead minnow, *Pimephales promelas*, exposed to feedlot effluent showed a range of responses including both feminisation of males and masculisation of females. Although the wetlands studied were not associated with feedlots, they were in an agricultural setting. A small herd of cattle grazed intermittently in the environs of the study wetlands although no supplementary feed or supplements were provided to the stock. Since the two control wetlands were in the same field, with equivalent access for cattle, and one had an excess of females and the other males, there is no evidence that pollution from stock, or farm chemicals (e.g., pesticides, herbicides, heavy metals) was responsible for either defeminisation or demasculation in this study.

Although the phenotypic sex ratio and size structure of the

populations of *G. holbrooki* were similar throughout the wetlands of our study and, therefore, not representative of endocrine disruption, there were differences in density and biomass that were consistent with the pattern of gonopodium deformity (used as a surrogate for endocrine disruption) found by Norris and Burgin (2011a, b). In each effluent chain, there was recovery from the presumed effects of endocrine disruption on the biomass and density of *G. holbrooki* in the second in the sequence of effluent streams. In addition, the control without suspected endocrine disruption (due to the lack of gonopodium deformity) had higher density and greater biomass than the alternative control wetland. The *G. holbrooki* population in this latter wetland was closer to those in the second in the stormwater wetland chain. Norris and Burgin (2011a, b) found that in both of these wetlands *G. holbrooki* had deformities of the gonopodium that they presumed were consistent with mild impairment by endocrine disruptors.

The results therefore do not support the null hypothesis that there was no effect of endocrine disruption on the population dynamics within the sampled wetlands. The findings were consistent with endocrine disruption having an impact on *G. holbrooki* biomass and density. Even within the fishes, reported to be more resilient to environmental challenges than other vertebrate groups due to sexual plasticity (Matthiessen 2000), and using a fish species that is widely accepted as pollution tolerant (Pyke 2005), it was observed that although the size structure and phenotypic sex ratio of *G. holbrooki* populations were apparently not challenged, density and biomass were diminished in the presence of endocrine disruption. However, without chemical evidence of endocrine disruption, ecology factors could not be discarded as an explanation for the results observed. For example, despite the demonstration that there was not a significant correlation between biomass and density, and turbidity, the level of turbidity was highest, and approaching double the level of other impoundments studied in the wetland where the highest biomass and density of *G. holbrooki* were resident. Turbidity may, therefore, influence *G. holbrooki* populations by constraining predator-prey interactions (Abrahams and Kattenfeld, 1997). Ecological factors such as a more direct measure of *G. holbrooki* prey than oxidation reduction potential, diet (Pen and Potter, 1991; Garcia-Berthou, 1999; Blanco *et al*., 2004), and predator and parasite/pathogen loads (Price *et al*., 1986; Hatcher *et al*., 2006), together with a quantitative assessment of endocrine disruption in *G. holbrooki* may have allowed for more convincing argument of causality. However, we consider this qualitative, preliminary study has laid the foundations for a more comprehensive study of the cause and effect relations of differences in population attributes of *G. holbrooki* resident in environments typically polluted with endocrine disruption chemicals.

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